Enhanced determination of pharmaceutical impurities through a temperature-responsive stationary phase in 2D-LC (TRLCxRPLC).

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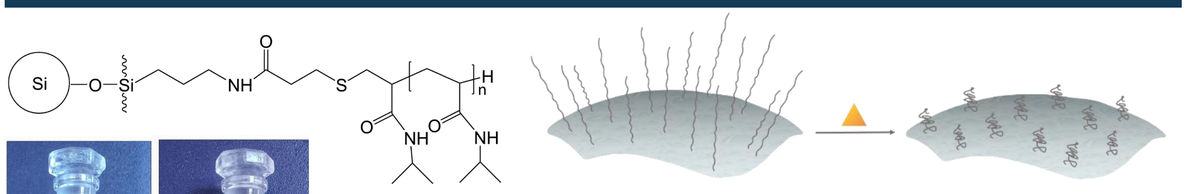
INTRODUCTION

Due to the increasing complexity of pharmaceutical drug formulations, assessment of the active pharmaceutical ingredients (API) and of their related impurities is becoming an increasingly more challenging task by conventional 1D-LC techniques. 2D-LC can thereby offer great benefits especially with respect to peak capacities, but common setups are often facing limitations due to solvent incompatibility issues, leading to peak breakthrough, peak broadening and consequently lower overall method sensitivity in the ^{2}D. A relatively new combination of separation modes, combining temperature-responsive with reversed-phase liquid chromatography (TRLCxRPLC) offers a relatively problem-free modulation with near to perfect peak refocusing at the 2D column head.[1] Such temperature-responsive phases thereby depict an adaptable hydrophobicity in aqueous solutions, and hence retention as a function of temperature.[2] A main benefit of the approach is that it forgoes the need for organic solvents in the mobile phase. This on the one hand allows for the transfer of high sample volumes from the first to the second dimension with near perfect peak refocussing,[1] and on the other hand facilitates more sensitive detection compared to conventional LCxLC approaches.

In this work, the possibilities offered by the unique combination of TRLCxRPLC are assessed for improved separation of synthetic mixtures of pharmaceutical compounds (steroids). Additionally, several column (core-shell) chemistries are assessed in the second dimension (EC-C18, PFP, Phenyl-Hexyl) to evaluate differences in selectivity.

TEMPERATURE-RESPONSIVE STATIONARY PHASE



hydrophilic ⟷ hydrophobic

Smart polymers change properties as a consequence of an environmental stimulus. Poly(N-isopropylacrylamide) (PNIPAAm) shows a change in polarity in aqueous solutions, upon varying the temperature. Below the so called lower critical solution temperature LCST, PNIPAAm is dissolved in aqueous solution and collapses when the temperature exceeds the LCST, displaying hydrophobic properties (Figure 1). When such polymers are coupled to a supporting material and packed in HPLC columns they allow obtaining reversed phase type separation in HPLC under purely aqueous conditions, inversed (downwards) temperature gradients can be used to control retention.

Figure 1. Illustration of the temperature-responsive effect of PNIPAAm.

EXPERIMENTAL

	Dimension 1	Dimension 2
Instrumentation	**Agilent 1290 Infinity II 2D-LC System**	
Column	TRLC column PNIPAAm based 100 x 2.1 mm, 5 µm, 100 Å	Agilent InfinityLab 120 Poroshell 1) EC-C18, 2) Phenyl-Hexyl, 3) PFP 50 x 3 mm, 2.7 µm, 120Å
Flow rate	0.1 ml/min	2.5 ml/min
Temperature	Temperature gradient: 0-30 min: 45ºC 30 min- end: 0ºC	Isocratic 50ºC
Mobile phase	(A) H2O+ 0.1 vol% FA	(A) H2O+ 0.1 vol% FA (B) ACN
Gradient	Isocratic (A)	Figure 2: Segment gradient 0 min - 16.90 min: 25-55% B 17 min - 26.90 min: 30-60% B 27 min - end min: 50-80% B Figure 3: Full gradient 20-80% B
Interface	2-position/8-port valve, tM=1 min, tg= 0.5 min, 120 µl loop	
Detection	DAD at 254 nm	

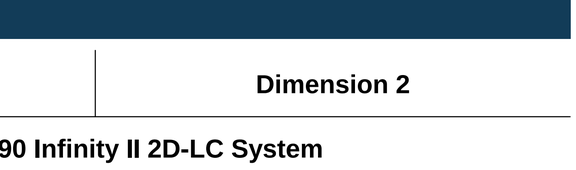
RESULTS AND DISCUSSION

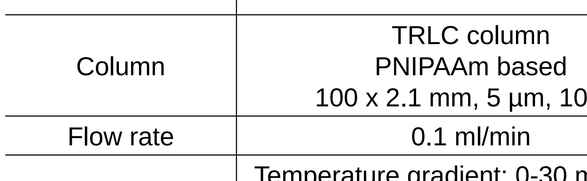
1) ORTHOGONALITY AND PEAK CAPACITIY

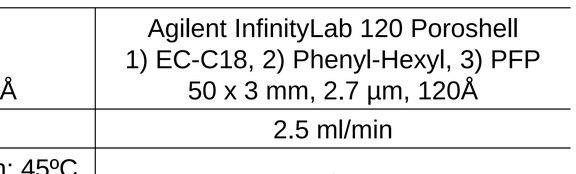
For this work, three different Poroshell columns were tested in the second dimension using 3 gradients to evaluate the ensuing differences in selectivity. The orthogonality was assessed using the Asterisk[3] method.[4] Note that also an adapted value was calculated, considering the entire separation space covered by the increasing gradients, and not restricted to the zones delimited by the first and last eluting peaks, as is more “conventionally” the case.

Repeatability was calculated for the retention factor (k) of each peak using the full gradient (TRLCxPFP) for n=3 consecutive measurements. The %RSD (k) for 1D was 0.72% and %RSD (k) in 2D 0.52%. The peak capacity was calculated following Li et al. [5] for the full gradient corrected for undersampling, resulting in a peak capacity of 848.

$$n'_{c,2D} = \frac{1n_c \times 2n_c}{\sqrt{(1+3.35(\frac{2t_c \times 1n_c}{1t_g})^2)}} = 848$$







	Full gradient		Shifted gradient		Segment gradient	
Orthogonality [%]	Asterisk	adapted	Asterisk	adapted	Asterisk	adapted
EC-C18	36	43	71	51	91	49
Phenyl-Hexyl	40	45	83	42	82	49
PFP	39	44	91	36	78	47

RESULTS AND DISCUSSION

2) SELECTIVITY

Retention on TRLC-based columns at high temperature is based on hydrophobic interactions. In Figure 2 inversed temperature-gradients (from 45° C to 0° C) have been used to elute strongly retained analytes faster. In the second dimension, three Poroshell 120 columns have been used and compared for their selectivity. The most polar column (PFP) is able to baseline separate all compounds more efficiently than the EC-C18 and the Phenyl-Hexyl column. Note, that the gradients have not been optimized for the specific column chemistries.

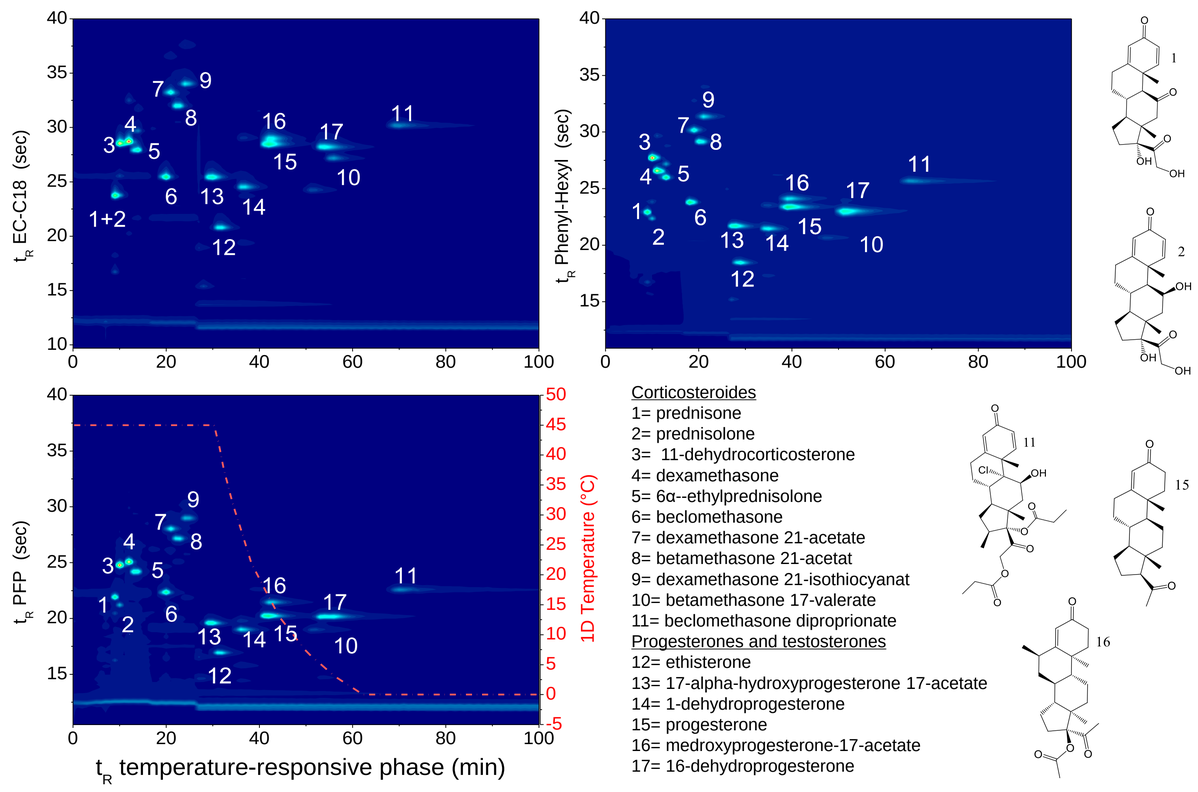


Figure 2. TRLCxRPLC of a steroid mixture (17 compounds) to compare selectivity of 3 different RP-stationary phases in the second dimension using the segment gradient: EC-C18, Phenyl-hexyl, PFP.

3) SENSITIVITY

In Figure 3 an API measurement is shown following ICH guidelines. All Impurities, present at a relative concentration of 0.05% (left) compared to the API were effectively detected. On the right, a second compound was adjusted to 2000 µg/ml, representing a combination therapy with multiple APIs in one drug product.

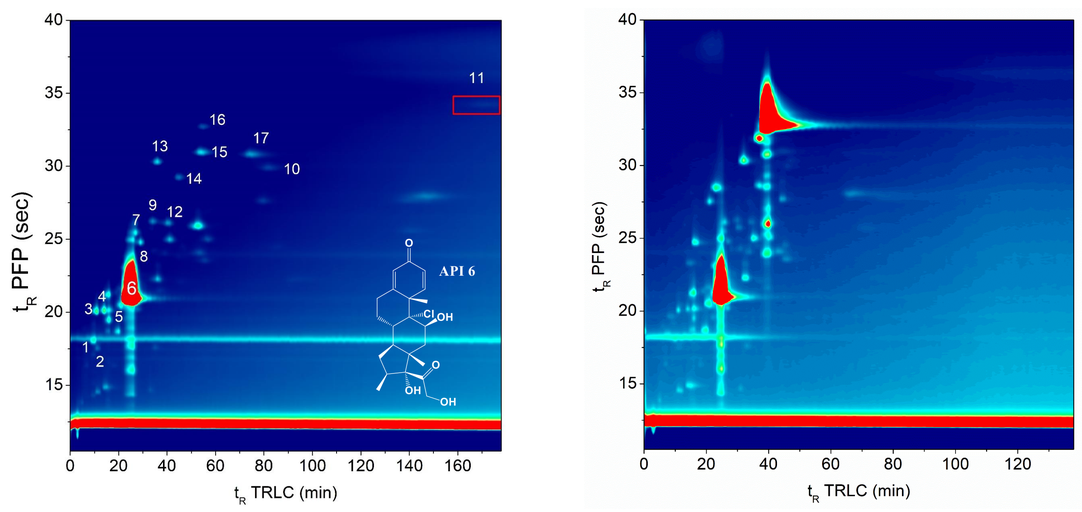


Figure 3. TRLCxRPLC separation of a test mixture of 17 structurally similar steroids (left) at an isocratic temperature of 45ºC, using the full gradient in the second dimension: impurities at 1 µg/ml, API at 2000 µg/ml, injection volume 6 µl; (right) two active pharmaceutical ingredients, an inverse temperature gradient in 1D (0-15 min: 45° C, 15.1-end: 0° C, using a full gradient in the second dimension (30-70% ACN). Impurities at 1 µg/ml, APIs at 2000 µg/ml, injection volume 3 µl

CONCLUSION

- It was shown, that impurity measurements can be performed with the required sensitivity to detect all 17 compounds by TRLCxRPLC. Highest orthogonality was achieved for the combination of TRLCxEC-C18 using the segmented gradients. Peak capacities can be further improved by optimizing sampling times.

Further developments

- More efficient ways need to be developed to implement faster temperature gradients in TRLC.

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